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14. ABSTRACT
The overall goal of this research project is to develop and implement a conceptually innovative strategy for down-regulating androgen receptor: the creation of bifunctional molecules that simultaneously bind to the androgen receptor and to bromodomain proteins Brd4. In this way, genes that are typically regulated by androgen receptor and glucocorticoid receptor will be extrinsically controlled. In this final year of funding, we completed the functional characterization of agonist and antagonist-based bi-functional recruiters. Importantly, we have a suite of recruiters that show context specificity. In the extension period of the work, we are completing RNAseq experiments to gain a complete understanding of the context specificity of this exciting class of molecules.

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INTRODUCTION

Androgen receptor (AR) is an allosterically-regulated transcription factor that binds to both the endogenous steroids testosterone and 5α-dihydrotestosterone and to a range of synthetic ligands. AR is central to prostate cancer pathogenesis and its reactivation is a hallmark of castration-resistant prostate cancer (CRPC), an aggressive and terminal illness for which there is no effective treatment. Despite this disease progression, most androgen-refractory prostate cancers continue to rely on AR for their survival; thus it remains an important therapeutic target.[1] Historically, approaches to modulate AR have focused on targeting the ligand-binding pocket with small molecules that sculpt the surface of the receptor in unique ways.[2] This indirect remodeling of the receptor binding surface results in the recruitment of different native binding partners. Although a powerful strategy, it has already been found that mutation of either binding surface (i.e. AR or cofactor) can occur, leading to, for example, antagonists that later become agonists. Even with the development of second-generation anti-androgens and small molecules that target sites other than the ligand-binding domain of AR, new small molecules are urgently needed that can suppress AR function and do not rely on allostery to elicit their effect.[3,4,5,6] In this project, we are developing an innovative alternative strategy to specifically steer and extend the repertoire of receptor-coregulator partnerships. By targeting ligand and substrate pockets in the receptor and coregulator complexes, we propose to bridge the two using novel small molecule bifunctional recruiters (Figure 1).

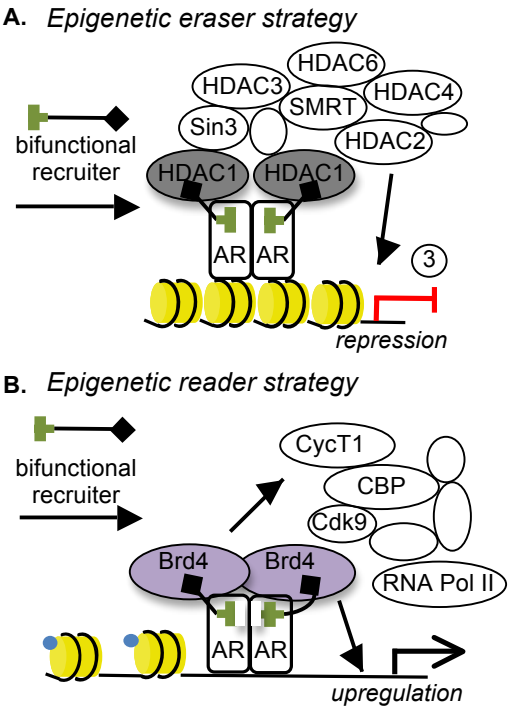


Figure 1. **A.** Epigenetic eraser strategy to block AR function. Bifunctional recruiters contain a high affinity AR ligand and an isoform-selective HDAC inhibitor (HDACi) to recruit corepressor complexes to AR. One functional consequence of this targeted recruitment could be deacetylation of chromatin, repressing transcription. **B.** Epigenetic reader strategy to enhance AR function. Bifunctional recruiters contain a high affinity bromodomain inhibitor to recruit BRD4 to AR regulated promoters. One functional consequence of this mode of targeted recruitment could be up-regulation, as depicted. However, transcriptional inhibition may be observed due to steric blockade at some genes.

Our original experimental plan focused upon one of the best-characterized mechanisms of transcriptional inhibition, the recruitment of large corepressor complexes that harbor histone deacetylase (HDAC) activity (Figure 1A).**Error! Bookmark not defined.**,7,8,9,10,11, 12 By appropriately linking HDAC inhibitors to high-affinity nuclear receptor ligands, we proposed to generate a new class of molecules that recruit transcriptionally repressing complexes to AR, a predicted consequence being the suppression of AR genomic function. As outlined in more detail in the body of this progress report, the targeted bifunctional molecules performed in vitro as designed and in cellular model systems; the preliminary studies supporting our model were published last year and, importantly, were chosen as the 'Best of Basic Research 2014' by the Endocrine Society (http://press.endocrine.org/bestofbasicresearch/2014). However, in cellular models of prostate cancer, no significant gene-specific or phenotypic effects were observed. We thus implemented the alternative strategy outlined in the original proposed work plan and illustrated in Figure 1B. In this strategy, we use the potent bromodomain inhibitor JQ1 to recruit BRD4 and thus extrinsically alter the transcriptional status of the targeted genes. As shown in the last funding period (months 13-24) this strategy was successfully implemented in a full-length nuclear receptor model, albeit not in an endogenous setting. In this final funding period we focused on three goals: (1) demonstration that altered transcriptional response is due to recruitment accomplished by the bifunctional molecule (Task 4); (2) assessment of agonist and antagonist-

based bifunctional recruiters in the context of endogenous genes; (3) genome-wide assessment of the best candidates via RNAseq in order to guide the design of the next generation of molecules. Because this has taken considerable additional effort than originally outlined in the Statement of Work, we requested and were granted a no-cost extension.

BODY

*Summary of **Task 1** goals:* The primary focus of Task 1 was the design and synthesis of bifunctional molecules consisting of high-affinity AR ligands linked to class-selective HDACi. Full in vitro characterization of the bifunctional molecules comprises this Task. Molecules with demonstrated affinity for both targets, comparable to unmodified inhibitors (within 2- to 4-fold), were carried on to *Task 2*.

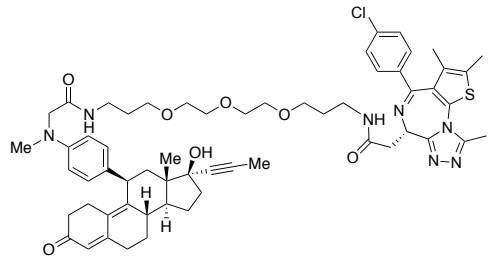
*Accomplishments of **Task 1**, Months 25-36:*

As outlined in the Year One and Year Two progress reports, all goals of Task 1 were accomplished in the first twelve months of the Project with the exception of the synthesis of PD106 conjugates of andarine. This was completed and the assessment of those molecules carried out in Task 2. A manuscript including preliminary results from Task 1 was accepted for publication and can be found in Appendix I (Jonas W. Højfeldt, Osvaldo Cruz-Rodríguez, Yasuhiro Imaeda, Aaron R. Van Dyke, James P. Carolan, Anna K. Mapp and Jorge A. Iñiguez-Lluhi, *Molecular Endocrinology* **2014** *28* 249-59).

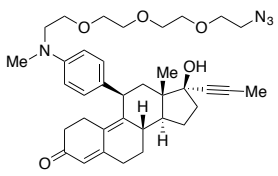
*Summary of **Task 2** goals:* Bifunctional molecules developed in the previous task will be examined in different cell lines (PC-3, LNCaP, VCaP) for their ability to modulate exogenous (luciferase) and endogenous (PSA, ERG) AR-driven reporters. Control experiments will also be performed to compare the effects of bifunctional molecules versus HDACi alone (i.e. HDACi without an AR-targeting moiety). Bifunctional recruiters that demonstrate cellular activity will be further examined as outlined in *Task 3*. If bifunctional molecules do not show efficacy in these assays, replacement of the HDACi recruitment moiety with an alternative inhibitor of chromatin modifying activities will be examined.

*Accomplishments of **Task 2**, Months 25-36*

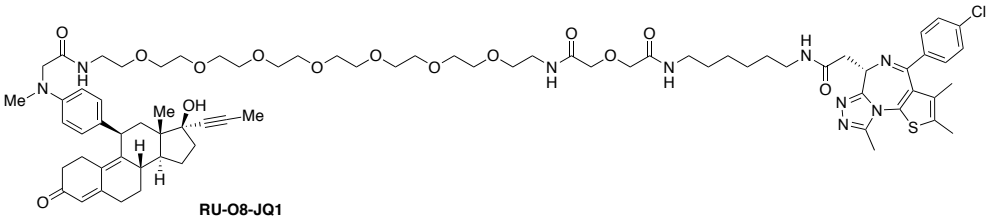
As outlined in the Year 2 progress report, assessment of the HDACi-based bifunctional recruiters revealed that any transcriptional effects due to recruitment were greatly attenuated. Our data from Task 1 and results from our model system (Appendix 1) illustrate the fundamental feasibility of extrinsic control of androgen receptor function through bifunctional recruiters. The primary difficulty with the HDACi-based molecules appears to be ineffective recruitment at the promoter level. Thus we sought to replace the HDACi moiety with an alternative recruiter that targets epigenetic reader proteins (i.e. bromodomains) instead of epigenetic erasers (i.e. HDACs). (S)-JQ1 is a highly potent and selective inhibitor of bromodomain 4 (BRD4) which is known to interact with critical components of the transcriptional machinery (e.g. CDK9, cyclin T1, RNA Pol II). Because our bifunctional molecules are constructed modularly, it was straightforward to conjugate existing synthetic intermediates with JQ1 using strategies developed for Task 1. Additionally, we established a productive collaboration with Professor James Bradner and Dr. Jun Qi (the discoverers of JQ1) at Dana Farber Cancer Institute; in order to facilitate meaningful functional and mechanistic analyses of our JQ1-based bifunctional molecules. In Year 2, we outlined the successful synthesis and analysis of antagonist RU486-based JQ1 conjugates, shown below in a reporter gene context. These were carried on to Task 3 and Task 4 experiments for this funding period (*vide infra*).



RU-O3-JQ1

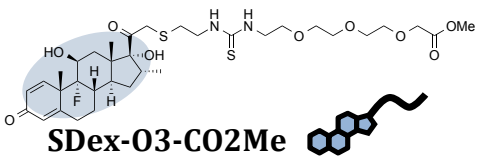


RU-O3

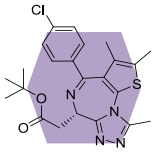


RU-O8-JQ1

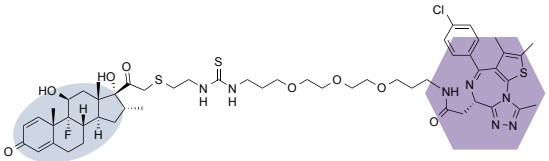
Agonist-based JQ1 bifunctional molecules. In addition to the RU-based JQ1 conjugates, in Year 3 we prepared and examined constructs based upon the agonist dexamethasone. As outlined in the previous Progress Report, the dynamic range for most androgen receptor-based transcriptional assays is sufficiently small that dissecting structure-function relationships for the bifunctional conjugates is difficult at best. Thus, we continued the use of glucocorticoid receptor (GR) as the model in which to test the bifunctional recruitment model. Given the modular nature of our design, lessons learned with GR will be readily portable to AR, our ultimate goal. Additionally, bifunctional molecules that target GR are likely to be therapeutically useful for prostate cancer. (21)



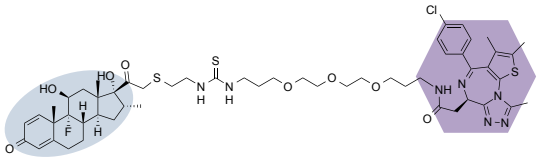
SDex-O3-CO2Me



(S)-JQ1



SDex-O3-(S)JQ1



SDex-O3-(R)JQ1

Summary of BRD4-targeting ligands. The ligands used in subsequent studies include unmodified (S)-JQ1, a monofunctional GR ligand-linker compound SDex-O3-CO2Me, a bifunctional SDex-O3-(S)JQ1 , and a bifunctional molecule conjugated to an inactive enantiomer of JQ1 termed SDex-O3-(R)JQ1.

Functional assessment of SDex-JQ1 bifunctional recruiters

To test these constructs, we have targeted full length human glucocorticoid receptor (hGR) as we and others have observed a more robust dynamic range in the transcriptional response. U2OS cells were transfected with a reporter plasmid bearing a consensus glucocorticoid response element (GRE) immediately upstream of a promoter driving luciferase expression and an expression plasmid coding for human GR. Cells were treated with either SDex-O3-CO2Me or SDex-O3-JQ1 (100 nM) for the indicated time. Transcriptional activity was determined by monitoring luciferase expression and displayed as fold activation over the levels of luciferase in vehicle-treated cells (Fig. 2). Both the monofunctional and bifunctional ligands acted as agonists of GR-mediated transcription, stimulating activity that increased with increasing incubation time. A 16-hour treatment produced the strongest transcriptional response and was chosen for further investigations into the activity of SDex-O3-JQ1.

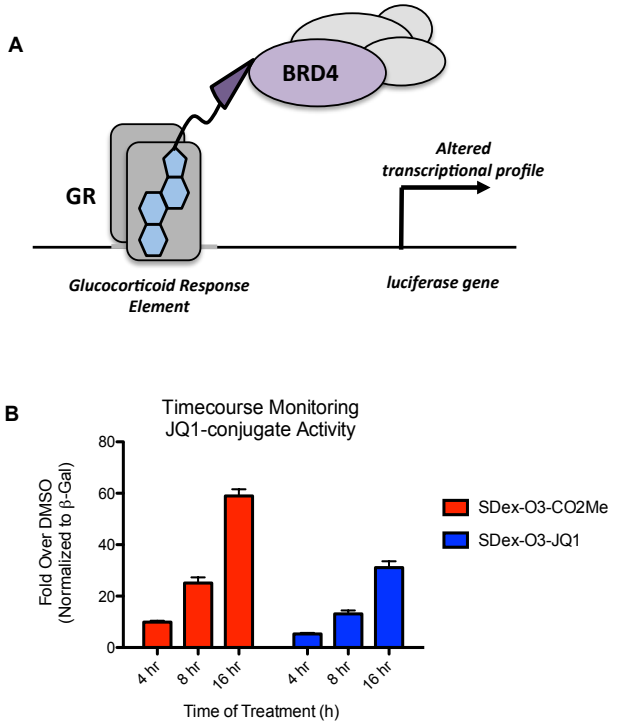


Figure 2. Recruitment of BRD4 to full-length GR. A reporter experiment was designed utilizing full-length GR (A). B: the activity of each GR-ligand was time dependent, with maximal observed activity occurring at 16 hours of treatment.

To examine effects at endogenous promoters, U2OS cells were transfected with luciferase reporter plasmids driven by different GR-regulated promoters and incubated with the indicated compound(s) for 16 hours. This measurement of transcriptional activity is displayed as a relative percentage, normalized to the activity produced by dosing with SDex-O3-CO2Me (100 nM) in Fig. 3A. Two additional controls were performed: a 'trans' dosing of equimolar SDex-O3-CO2Me and biotinylated (S)-JQ1 [(S)-JQ1-biotin] as well as a 'squelch' dosing with SDex-O3-JQ1 and an excess (10 μM) of (S)-JQ1-biotin. The trans addition components were chosen to ensure that transcriptional outcomes caused by SDex-O3-JQ1 are not simply additive or synergistic responses, provoked independently by each moiety in the bifunctional molecule. The squelch dosing is included to confirm that effects are induced through BRD4 recruitment. As expected, SDex-O3-CO2Me acts agonistically to activate GR-driven transcription, while co-dosing with (S)-JQ1-biotin results in a suppression of transcription at high (1 μM) concentrations, but otherwise minimally affects the activity of SDex-O3-CO2Me. Increasing concentrations of SDex-O3-JQ1 result in a bell-shaped activity curve, initially increasing at low concentrations but declining with higher concentrations of compound; at the highest dose (1 μM), activity levels are approximately 50% of the maximum stimulation level (produced at 10 nM). The co-addition of excess (S)-JQ1-biotin increases activity, albeit not to the same level as seen with the trans addition treatment. The suppression of activity seen with SDex-O3-JQ1, paired with the observation that free (S)-JQ1-biotin competes with suppression, suggests that the recruitment of BRD4 is interfering with GR-mediated transcription. **As outlined in Task 4, these and other data indicate that bifunctional recruitment is the driver for the altered gene expression patterns observed, an important finding.** Thus, the SDex-based bifunctional molecules in addition to the RU486-based constructs were carried forward to the Task 3 experiments.

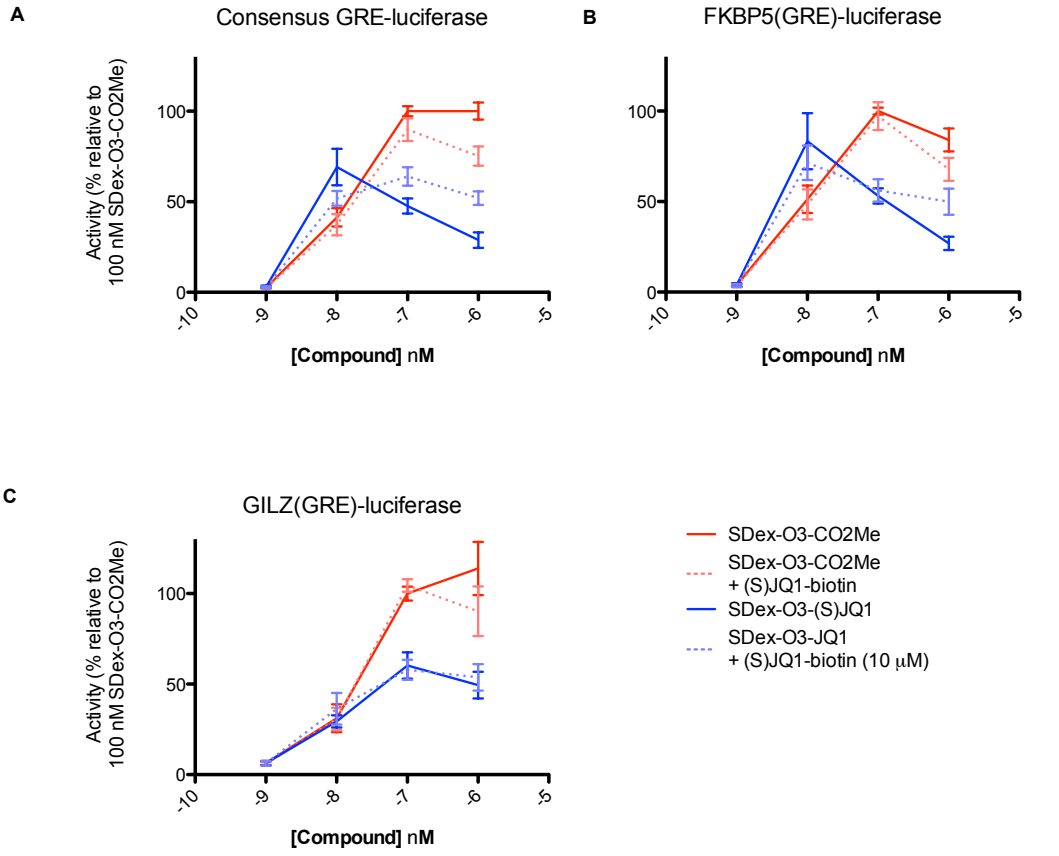


Figure 3 Utilizing alternative GREs in reporter experiments. Full-length GR was utilized in a transcriptional reporter experiment, with a reporter plasmid using a consensus GRE (A), *FKBP5* GRE (B), or *GILZ* GRE (C).

***Summary of* Task 3 goals**. The overall goal of Task 3 is to quantitatively define the unique properties of the bifunctional recruiters as well as assess their mechanism of action. In the original Statement of Work, the subtasks described were centered around HDACi-based recruiters, examining alterations in acetylation patterns, for example. Because we moved on to bromodomain-targeting bifunctional recruiters, the details of the experiments shifted to the definition of the unique properties of these specific molecules. In the Year 2 progress report, we described the initial characterization of RU486 (antagonist)-based bifunctional recruiters. In months 25-36 we completed an examination of agonist (SDex)-based JQ1 recruiters at a suite of endogenous genes with endogenous full-length receptor in comparison with controls and, finally, the genome-wide analysis to determine the context-specificity of the molecules. Additionally, we found that RU486-based bifunctional recruiters are gain-of-function molecules, able to override the intrinsic repressive effects of RU486.

Accomplishments of* Task 3 *goals, months 25-36

SDex-JQ1 bifunctional recruiters at endogenous promoters To investigate the effects of bifunctional molecule treatment on endogenous gene expression, U2OS cells were transfected with an expression plasmid for human GR and treated with the indicated compound(s). Following treatment, cells were lysed, total RNA isolated, and the indicated transcript was quantified relative to the levels in vehicle-treated cells. The transcriptional activity is displayed as fold activation in Fig. 4. The relative transcript levels of *S100P* (Fig. 4A), *FKBP5* (Fig. 4B), and *GILZ* (Fig. 4C) were determined. SDex-O3-CO2Me treatment activated the transcription of each of these three GR-target genes. Unlike in reporter experiments, co-dosing with (S)-JQ1-biotin did not appreciably alter the activity of SDex-O3-CO2Me; this may be a byproduct of a shorter dosing time in RNA quantification studies, though the transcriptional response to both SDex and (S)-JQ1 is rapid. In comparison to SDex-O3-CO2Me, SDex-O3-JQ1 weakly activated transcription of the *S100P* gene (Fig. 4), raising levels to approximately 25% of the maximum level induced by SDex-O3-CO2Me. Consistent with the hypothesis that BRD4 recruitment inhibits GR activity, the addition of excess (S)-JQ1 allows SDex-O3-JQ1 to activate *S100P* transcription to similar levels as SDex-O3-CO2Me. SDex-O3-JQ1 induced a similar, but less pronounced, effect in activating transcription of the *FKBP5* gene to approximately 60% of the level induced by SDex-O3-CO2Me (Fig. 4). Again, addition of excess (S)-JQ1 squelches the suppressed agonism by SDex-O3-JQ1, raising activity to a level comparable with SDex-O3-CO2Me. Interestingly, the transcriptional response at the *GILZ* gene was even more disparate; treatment with SDex-O3-JQ1 alone or in tandem with (S)-JQ1 produced a near identical activation of *GILZ* transcription. While further analysis of additional targets is necessary prior to making conclusions, the wide range in activity induced by the bifunctional glucocorticoid may point to the context specificity of the bifunctional recruiters.

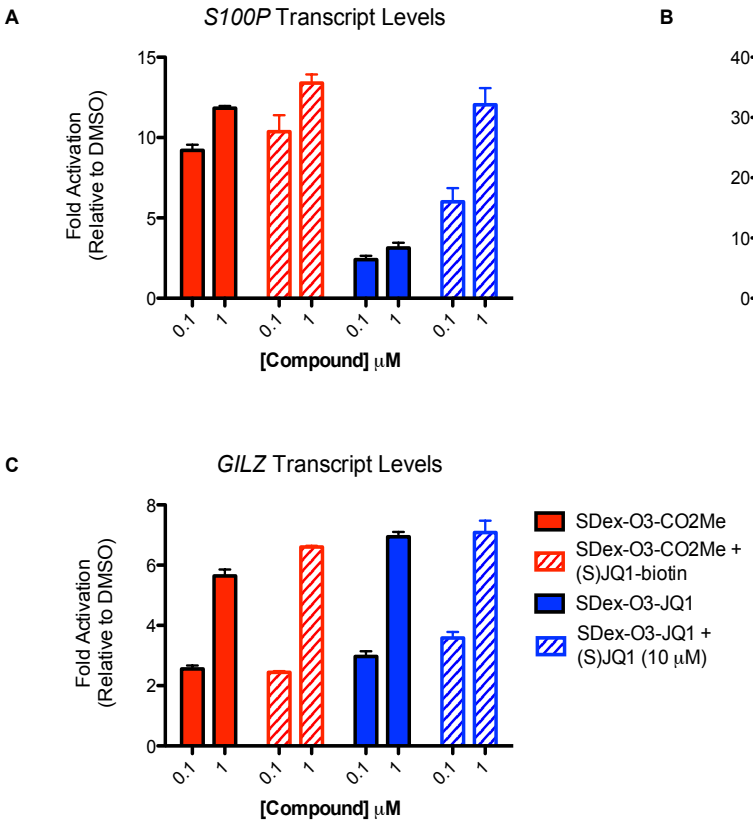


Figure 4 Effects of BRD4 recruitment on endogenous gene transcription. The effects of the designed ligands on the transcription of *S100P* (A), *FKBP5* (B), and *GILZ* (C) in transfected U2OS cells are determined through RT-qPCR analysis. Transcript quantification was normalized to the housekeeping gene *RPL19* and depicted as fold activation, relative to DMSO control, using the $\Delta\Delta C_T$ method.

The human adenocarcinoma lung epithelial cell line A549 expresses endogenous GR and is commonly used as a model line for the study of GR actions and activity. The effects of SDex-O3-JQ1 treatment on transcriptional activity in A549 cells was investigated to determine if similar patterns are displayed in cells expressing endogenous levels of GR. Comparing the transcription of *S100P* and *GILZ* provided the starkest difference in activity mediated by SDex-O3-JQ1 and provided a template for further studies in A549 cells and, in the future, in PC3 and other prostate cancer systems. Following treatment with the indicated compounds, A549 cells were lysed, total RNA was isolated, and the indicated transcripts were quantified. Transcriptional activity is displayed as fold activation relative to transcript levels in vehicle-treated cells (Fig. 5). The activation patterns of *S100P* (Fig. 5A) in A549 cells resemble the profile produced in transfected U2OS cells. Treatment with SDex-O3-CO2Me in the absence or presence of (S)-JQ1 produced a strong agonistic response, while treatment with the bifunctional SDex-O3-JQ1 activated transcription to approximately 50% of the level induced by the monofunctional glucocorticoid. As observed before, co-treatment of SDex-O3-JQ1 with an excess of (S)-JQ1 produces transcriptional activity nearly identical to SDex-O3-CO2Me, implying that (S)-JQ1 is capable of competing away BRD4 recruitment and causing SDex-O3-JQ1 to act as its parent, monofunctional ligand. Similarly, the pattern of *GILZ* transcription (Fig. 5B) resembled the activities produced in transfected U2OS cells, wherein each of the described compounds and combinations produced nearly identical responses.

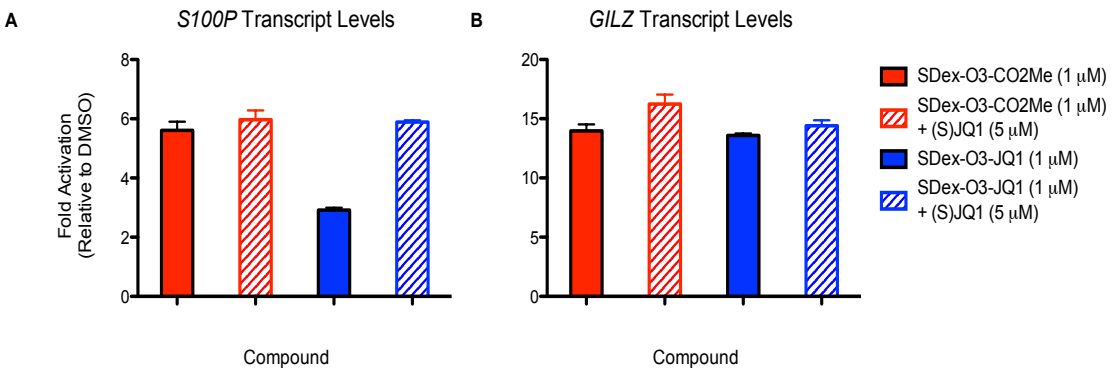


Figure 5 Transcriptional modulation in A549 cells. The effects of the designed ligands on the transcription of *S100P* (A) and *GILZ* (B) in A549 cells are determined through RT-qPCR analysis. Transcript quantification was normalized to the housekeeping gene *RPL19* and depicted as fold activation, relative to DMSO control, using the $\Delta\Delta C_T$ method.

Gain of function bifunctional recruiters based upon RU-486. In the previous funding period (Year 2) we demonstrated that RU486-JQ1 conjugates functioned in reporter contexts. More recently we examined a larger group of genes to assess how broadly those effects extend and if they are indeed dependent upon an interaction with Brd4. U2OS cells were transfected with an expression plasmid coding for hGRα and a luciferase reporter plasmid driven by a promoter containing a consensus GRE (Fig. 6), a GRE from the *FKBP5* sequence (Fig. 6B), or a GRE from the *GILZ* promoter (Fig. 6C). The 'trans' addition employed in this set of trials contained a biotinylated version of (S)-JQ1, acknowledging its utility as a better mimic of the effects of synthetic conjugation on (S)-JQ1. Following treatment, cells were lysed and luciferase levels were quantified and displayed relative to those in vehicle-treated cells. Regardless of the GRE employed, the monofunctional ligand RU-O3-N3 failed to appreciably elevate transcription of luciferase in both the absence and presence of (S)-JQ1-biotin. However, the bifunctional ligand produced a dose-dependent activation of transcription in all three systems, significantly raising transcriptional levels at concentrations as low as 0.1 μM. This was an exciting observation that RNAseq experiments will be critical for contextualization.

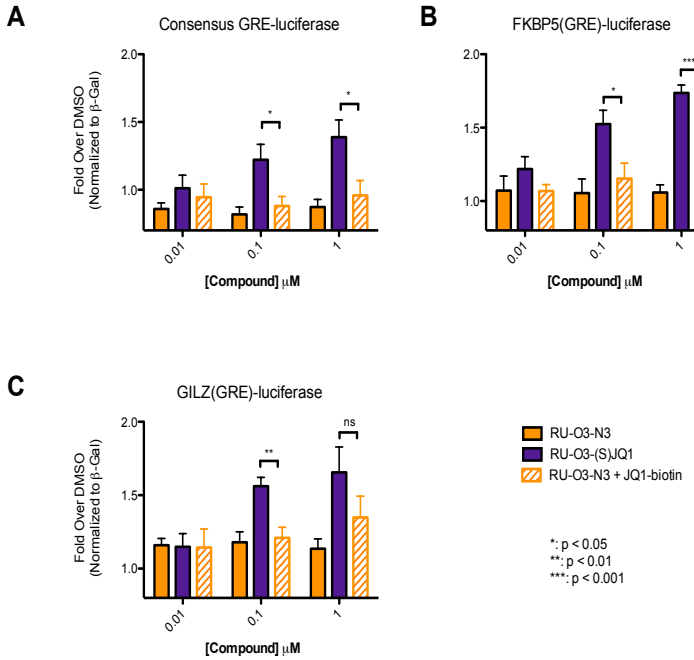


Figure 6. Transcriptional activity of full length human GR in the presence of bifunctional recruiters. Full-length GR was utilized in a transcriptional reporter experiment, with a reporter plasmid using a consensus GRE (A), *FKBP5* GRE (B), or *GILZ* GRE (C).

Assessing context specificity via RNAseq The data described in the previous section, in addition to the results from the Year 2 progress report, indicate that the activity patterns of the bifunctional molecules are similar in different cell lines and, further, that there is considerable context specificity from gene to gene. In other words, a given bifunctional recruiter may function as an activator, a repressor or have no effect at any given gene and, thus far, we have seen no conceptual principles emerge that enable prediction of these patterns. Based upon this and other lines of evidence, we elected to carry out an RNAseq experiment in glucocorticoid-sensitive (MM1S) and resistant (MM1R) matched cell lines in order to obtain a complete picture of context specificity; further supporting this choice of cell lines is the expertise of our collaborator Dr. James Bradner with this specific model system and the wealth of data for JQ1 and derivatives. Briefly, cells were treated with 1 or 10 μM bifunctional molecules for 4 hours followed by isolation of total mRNA by standard methods. We are currently awaiting the data and full analysis from Dana Farber.

Summary of* Task 4 *goals The primary goal of Task 4 was to complete thorough mechanistic investigations of the bifunctional recruiters to demonstrate engagement of both targets. The experiments outlined in the original SOW were centered around HDACi-based recruiters. As the design of the molecules changed to include BRD4-targeting molecules, the specific experiments altered but the goal of demonstrating the bifunctional recruitment mechanism remained.

Summary of* Tast 4 *accomplishments As illustrated below, a variety of biochemical and cellular experiments demonstrate that in the context of agonist-based bifunctional molecules, binding to the nuclear receptor and to BRD4 is accomplished by the bifunctional molecules and, further, that this is important for function. As will be discussed in more detail in the final report, for antagonist-based structures such as RU-486, the attenuated binding affinity that results from even carefully designed conjugation is likely perturbing the system enough that transcriptional effects are minimal. Our data overall suggest that if this can be overcome (and it should be through the synthesis of analogs), the bifunctional recruitment strategy will be readily portable to other nuclear receptors.

Bifunctional molecules interact with receptor and BRD4 in the cellular milieu To first determine if the designed SDex-O3-(S)JQ1 is capable of dimerizing GR and BRD4 in the absence of DNA-binding, ligand-induced coimmunoprecipitation experiments were performed. The human osteocarcinoma U2OS cell line lacks detectable GR expression and was chosen for this study. U2OS cells were transfected with an expression plasmid coding for a multiple myc-tagged version of human GR (hGR-myc6) and incubated with the indicated compound. Samples were subsequently lysed and incubated with magnetic beads coated in BRD4-recognizing antibody (αBRD4). Bound proteins were eluted and analyzed by Western blot for the presence of hGR-myc6 using a myc-recognizing antibody (Fig. 7). SDex-O3-(S)JQ1 induced the coimmunoprecipitation of GR with BRD4. Neither the vehicle (DMSO) or linker (SDex-O3-CO2Me) incubated-samples result in the coimmunoprecipitation of hGR-myc6, precluding the possibility of a non-specific interaction between GR and BRD4. Additionally, the inactive diastereomer, SDex-O3-(R)JQ1, was incapable of coimmunoprecipitating hGR-myc6, strongly supporting the notion that the observed interaction is being specifically modulated through the interaction of each portion of the bifunctional molecule with its target binding pocket.

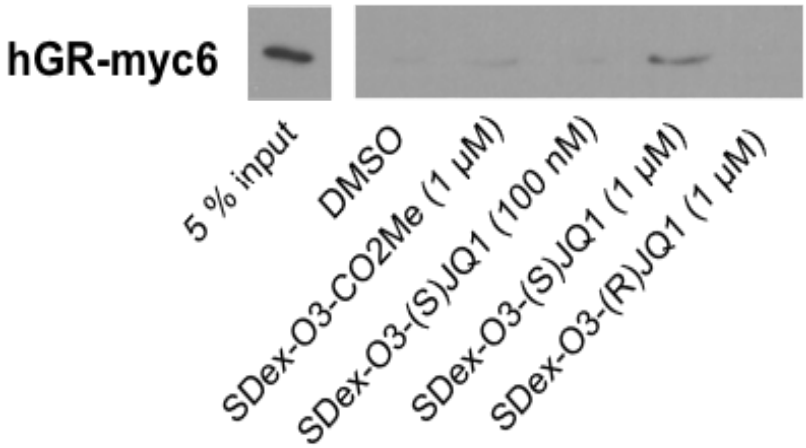


Figure 7 Dimerization of hGR-myc6 and BRD4. Co-immunoprecipitation experiments were performed to detect the ability of the bifunctional molecule to dimerize hGR-myc6 and BRD4. Cellular lysates were incubated with bifunctional molecule and then with beads pretreated with a BRD4 recognition antibody. The eluted complexes were then analyzed by western blotted using αmyc.

SDex-JQ1 function is dependent upon GR LBD and BRD4 To investigate the effects of agonist-facilitated BRD4 recruitment on transcription, we employed a traditional three-hybrid experiment (depicted in Fig. 7) as we had used previously with the RU486-based molecules (Year 2 progress report). This arrangement additionally allows us to confirm that SDex-O3-JQ is capable of chemically dimerizing GR and BRD4 in a cellular system. HeLa cells expressing endogenous levels of BRD4 were transfected with a luciferase reporter plasmid bearing five Gal4 DNA-binding sites and an expression plasmid for a Gal4-GR(LBD) chimera and treated with the indicated compounds. The resulting activation of transcription is displayed in Fig. 8B as a fold activation of luciferase produced over the levels in vehicle-controlled cells. As expected, the monofunctional SDex-O3-CO2Me acted as an agonist in this system, likely activating transcription through its induced rearrangement of the GR(LBD) AF2 domain. As was seen with the recruitment of VP16-FKBP, the bifunctional SDex-O3-JQ1 acts as a 'superactivator' of transcription, stimulating the expression of luciferase approximately 3-fold higher than the maximum activity displayed by SDex-O3-CO2Me. A squelching experiment was subsequently performed with JQ1 to determine if BRD4 recruitment is the likely mechanism for producing this activity level. HeLa cells were transfected as above and treated with SDex-O3-JQ1 (1 μM) along with increasing levels of either (S)-JQ1 or inactive (R)-JQ1. As seen in Fig. 8C, increasing levels of (S)-JQ1 suppressed the transcriptional activity of SDex-O3-JQ1 in a dose-dependent fashion, with high concentrations of (S)-JQ1 (10 μM) suppressing activity approximately 70%. However, co-treatment with (R)-JQ1 did not produce this effect. This observation supports the hypothesis that BRD4 recruitment is causing the marked difference in activity between SDex-O3-CO2Me and SDex-O3-JQ1; however, (S)-JQ1 is capable of interfering with dexamethasone-induced transcription (unpublished observations) and we are completing additional experiments to be able to draw firm conclusions.

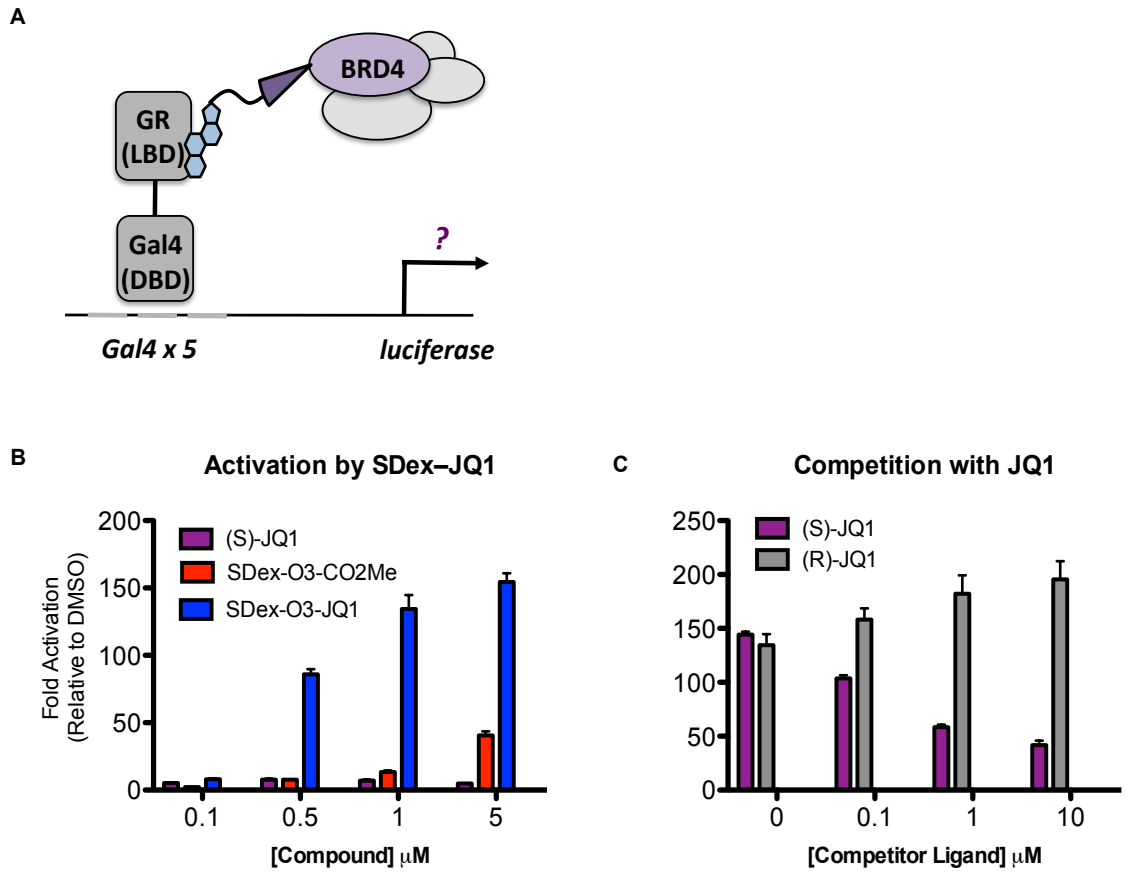


Figure 8 Mammalian three-hybrid targeting an endogenous protein. (A) A three-hybrid experiment, using a Gal4-GR(LBD) chimera, relying on endogenous BRD4 recruitment to drive activity was constructed. (B) The monofunctional GR ligand acted as a partial agonist, while bifunctional ligand strongly and potently activated transcription. (C) The enhanced activity of the bifunctional molecule was dependent on BRD4 recruitment, as determined by squelching experiments.

KEY RESEARCH ACCOMPLISHMENTS

• Identified an agonist-based scaffold that extrinsically regulates full-length, native nuclear receptor at endogenous promoters in more than one cellular context. Importantly, this scaffold shows considerable context specificity.

• Biochemical and cellular experiments (Task 4) support a model in which the agonist-based bifunctional recruiters function through interaction with both binding partners. This validates the overall design.

• Our new data illustrate that we can create gain-of-function bifunctional recruiters, in which the recruitment of Brd4 overrides the intrinsic repressive effects of RU486.

• See 2 Prism files I attached (5AVD_102 Activation and 5AVD_102 Squelch). This data is the same set of experiments as shown in Figure 2.13 but with RU-based compounds. We see really nice activation and nice squelching with (S)-JQ1. Your third bullet point could be gain-of-function bifunctional recruiters. The RU data shows that BRD4 recruitment in specific contexts can activate instead of repress (an idea introduced in Fiture 1B). Perhaps not surprising because we've already seen context is important with the SDex

compounds. I can't remember but if Jay is also trying the RU compounds we can say we look forward to mining this data to discern contextual patterns of activation/repression.

REPORTABLE OUTCOMES

• A manuscript outlining the findings of Task 3 and 4 is currently in preparation: Van Dyke, A.; Carolan, J.P.; Qi, J.; Pawlik, J.; Bradner, J. Mapp, A.K. *Manuscript in preparation.*

• The Task 2 and 3 data have been presented by (former) postdoctoral fellow and current Assistant Professor Dr. Aaron Van Dyke at the 2014 Bioorganic Chemistry Gordon Research Conference. Graduate student JP Carolan has presented this work at the 2014 Life Sciences Institute Poster session (Nov 2014), the 229th American Chemical Society National Meeting in San Francisco, the 2014 Vaughn Symposium at the University of Michigan.

CONCLUSIONS

The overall goal of this research project is to develop and implement a conceptually innovative strategy for down-regulating androgen receptor: the creation of bifunctional molecules that simultaneously bind to the androgen receptor and to chromatin modifying complexes. In this way, AR function can be extrinsically controlled. In this third year of funding, we made critical progress towards this goal with the demonstration that targeting an epigenetic reader, BRD4, is a successful strategy through the use of agonist-based ligands. Additionally, we demonstrated that the mechanism of action is dependent upon both binding partners. As we analyze the genome-wide analysis of our RNAseq experiments, we will derive a model on which to base bifunctional molecules with better properties for androgen-receptor targeting.

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Appendix 1: Abstracts of presentations

November, 2014 - Life Sciences Institute Annual Poster Session (Univ of Michigan, Ann Arbor, MI) - title: Alternative Modulation of Glucocorticoid Receptor Activity

Abstract: Nuclear receptors (NRs) such as the glucocorticoid receptor (GR) are ligand-inducible transcription factors that regulate gene expression by recruiting protein complexes to DNA. Misregulation of this process has been implicated in a range of disease states. Historically, NRs have been therapeutically modulated by small molecules that *allosterically* recruit coregulator complexes that up- or down-regulate gene expression. Alternatively, bifunctional molecules that *directly* control coregulator recruitment would be excellent tools to provide new profiles of gene expression. Bifunctional molecules that target the GR and the lysine reader protein BRD4 have been synthesized and are being evaluated for activity *in vitro*, with the results being discussed here.

August, 2014 - 249th ACS National Meeting and Exposition (San Francisco, CA) - title: Alternative Modulation of Glucocorticoid Receptor Activity Using Bifunctional Small Molecules

Abstract: Nuclear receptors (NRs) such as the glucocorticoid receptor (GR) are ligand-inducible transcription factors that regulate gene expression by recruiting protein complexes to DNA. Misregulation of this process has been implicated in a range of disease states. Historically, NRs have been therapeutically modulated by small molecules that *allosterically* recruit coregulator complexes that up- or down-regulate gene expression. Alternatively, bifunctional molecules that *directly* control coregulator recruitment would be excellent tools to enhance control of gene expression. Bifunctional molecules that target the GR and the lysine reader protein BRD4 have been synthesized and are being evaluated for activity *in vitro*, with the results being discussed here.

July, 2014 - Victor Vaughan Symposium (Univ of Mich, Ann Arbor, MI) title: Alternative Modulation of Glucocorticoid Receptor Activity Using Bifunctional Small Molecules

Abstract: Nuclear receptors (NRs) such as the glucocorticoid receptor (GR) are ligand-inducible transcription factors that regulate gene expression by recruiting protein complexes to DNA. Misregulation of this process has been implicated in a range of disease states. Historically, NRs have been therapeutically modulated by small molecules that *allosterically* recruit coregulator complexes that up- or down-regulate gene expression. Alternatively, bifunctional molecules that *directly* control coregulator recruitment would be excellent tools to enhance control of gene expression. Bifunctional molecules that target the GR and the lysine reader protein BRD4 have been synthesized and are being evaluated for activity *in vitro*, with the results being discussed here.